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Effects of reducing dietary starch content by replacing barley grain with wheat dried distillers grains plus solubles in dairy cow rations on ovarian function

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ABSTRACT

Our objective was to evaluate the effects of dietary starch content, altered by partial substitution of dietary grain with wheat dried distillers grain with solubles (DDGS), on the interval from calving to first ovulation, concentrations of hormones and metabolites in plasma and follicular fluid, and granulosa cell gene expression in preovulatory follicles. Sixty lactating dairy cows were assigned to 1 of 2 diets from calving until 84 d postpartum. Diets were formulated to contain either 17.3% rolled barley grain (29.2% starch) or 17.2% wheat DDGS (19.1% starch), with 43.0% barley silage and 21.6% rolled corn grain as the other major ingredients (dry matter basis). Transrectal ultrasonography was performed twice weekly to monitor ovarian dynamics from 7 ± 2 d postpartum until ovulation or until 56 d in milk, whichever occurred earlier. Plasma concentrations of insulin and insulin-like growth factor-1 (IGF-1) were determined in all 60 cows, and that of glucose, fatty acids, and urea in a subset of 24 cows, representing those in which the first ovulation occurred spontaneously within 5 wk postpartum. Estradiol (proestrus) and progesterone (12 d postovulation) in plasma were also measured. Concentrations of insulin, IGF-1, glucose, fatty acids, and urea were determined in follicular fluid (wk 9), and the expression of LH receptor, estrogen receptor β, cytochrome P450 aromatase, and plasma type glutathione peroxidase genes measured in granulosa cells obtained from the preovulatory follicles at wk 9 postpartum in the subset of 24 cows. Diets did not alter the interval from calving to first ovulation (32.3 ± 2.5 d), but a significantly lower proportion of cows on the DDGS diet (20%) ovulated multiple (≥2) follicles at the first ovulation than those on the barley grain diet (40%). The incidence of multiple ovulations tended to be lower at first insemination (10 vs. 21% for cows fed DDGS and barley grain diets, respectively). Mean plasma concentration of insulin was higher in cows fed the barley grain diet (2.5 vs 1.6 IU/mL), and a diet by time interaction was noted, with cows on the barley grain ration having higher insulin from wk 6 to 12 postpartum; however, mean plasma IGF-1 concentration did not differ between dietary groups. In the subsets, mean plasma concentrations of metabolites or estradiol and progesterone were not affected by diet, parity, or diet by parity interactions. Cows on the DDGS diet had lower concentrations of IGF-I (69 vs. 108 ng/mL) and higher fatty acids (222 vs. 149 mEq/L) in the follicular fluid obtained from preovulatory follicles. Diet, parity, and diet by parity interactions did not affect the concentrations of insulin, glucose, urea, estradiol, and progesterone in follicular fluid. Diets did not alter the expression profiles of *LHr*, estrogen receptor β, *CYP19*, and *GPx3* genes in granulosa cells. In summary, diets did not affect the interval from calving to first ovulation or granulosa cell gene expression. However, reducing dietary starch content by a partial replacement of dietary grain with wheat DDGS increased fatty acids in follicular fluid and reduced the concentrations of insulin in plasma, IGF-1 in follicular fluid, and the incidence of multiple ovulations.

Key words: starch, dried distillers grains with solubles, lactating dairy cow, multiple ovulations, follicular fluid

INTRODUCTION

Lactating dairy cows are often in a state of negative energy balance for up to 6 to 8 wk postpartum, which has been associated with an increase in the interval from calving to first ovulation (Butler and Smith, 1989) and a decrease in conception rates (Butler, 2000). Mobilization of fat reserves associated with negative energy balance increases plasma concentrations of fatty

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acids and may interfere with availability of hormones such as insulin and IGF-1 in early lactation (Bossaert et al., 2008). Insulin and IGF-1 have been shown to play critical roles in follicular cell proliferation and steroidogenesis in vitro (Spicer and Echternkamp, 1995). Armstrong et al. (2001) reported that heifers fed a high-energy (816 kJ/kg) diet had higher plasma concentrations of insulin and IGF-1 compared with those fed a low-energy (408 kJ/kg) diet. The same research group (Armstrong et al., 2002) also reported that dietary-induced changes in circulating insulin and IGF-1 concentrations have a direct effect on the steroidogenic potential of bovine granulosa cells obtained from small follicles, where cows were given either maintenance or twice maintenance diets during the experimental period. In addition, raising the circulating concentrations of insulin by increasing dietary starch content from 10 to 26% resulted in a greater proportion of cows ovulating before 50 d postpartum (Gong et al., 2002). In a more recent study (Dyck et al., 2011), resumption of cyclicity occurred sooner in cows fed diets containing 27% starch compared with those fed diets containing either 25 or 23% starch, although plasma concentrations of insulin and IGF-1 did not differ among dietary treatments. Cows ovulating sooner postpartum are more likely to conceive to first insemination (Butler, 2000; Ambrose and Colazo, 2007). Therefore, feeding high-starch diets during the early postpartum period could be a strategy to improve reproductive performance of lactating dairy cows. However, an association between high-starch diets and increased incidence of multiple ovulations has been reported (Dyck et al., 2011). Double ovulation is the main reason for twin pregnancies in cattle (Wiltbank et al., 2000) and twinning is an undesirable outcome as it often increases the risk of postpartum disease, increases average days open and services per conception during the subsequent lactation, increases the probability of culling, and reduces the productive lifespan of cows calving twins than that of cows calving singletons (Andreu-Vázquez et al., 2012).

The expansion of ethanol fuel production in western Canada in recent years has increased the supply of ethanol by-products, such as wheat dry distillers grain with solubles (**DDGS**). Due to its increased availability at a reasonable cost and high NDF of about 37.4%, DDGS is being used as an alternative feedstuff in postpartum dairy cows (Zhang et al., 2010). Whereas DDGS can be used to partially substitute grain, the effects of including wheat DDGS in dairy rations, to reduce dietary starch, on ovarian function of lactating dairy cows have not been investigated.

We hypothesized that reducing dietary starch content by replacing barley grain with wheat DDGS would affect plasma concentrations of insulin, IGF-1, and ovarian function, and alter the intrafollicular milieu. Specific objectives were to evaluate the effects of dietary starch content, altered by partial substitution of dietary grain with wheat DDGS, on the interval from calving to first ovulation, concentrations of hormones and metabolites in plasma and follicular fluid, and gene expression in granulosa cells of preovulatory follicles.

MATERIALS AND METHODS

Animals and Diets

The study was conducted in a tiestall barn at the University of Alberta. Experimental protocols were approved by the Animal Care and Use Committee for Livestock at the University of Alberta and animals were cared for in accordance with the requirements of Canadian Council on Animal Care (1993).

Sixty lactating Holstein cows (22 primiparous and 38 multiparous) were assigned to this study. Cows were individually housed in a tiestall barn and let out for approximately 2 h of exercise on weekdays. Body condition score was determined based on a 5-point scale (Wildman et al., 1982) 1 wk before expected date of calving. Cows, blocked by parity and date of calving, were assigned to 1 of the 2 experimental diets starting on the day of calving until 84 DIM (Figure 1). Diets were formulated according to NRC (2001) to meet the requirements of a 650-kg lactating cow producing 45.0 kg of milk/d with 3.5% milk fat and 3.0% milk protein. Following calving, cows were assigned to 1 of the 2 experimental diets containing either 17.2% wheat DDGS or 17.3% rolled barley, and 43.1% barley silage. Ingredient composition of the diets has been previously published in a companion paper (Sun and Oba, 2014; Table 1). Cows were offered the assigned diets once daily as TMR at 105 to 110% of the expected intake at 0730 h and had access to water ad libitum. The amounts of feed offered and refused were recorded individually to assess daily DMI for each cow. Cows were milked daily in their stalls at 0400 and 1600 h, and daily milk production was recorded.

Ultrasonography and Timed AI

Transrectal ultrasonography (Aloka-500V scanner equipped with a 7.5-MHz linear transducer, Aloka Co., Tokyo, Japan) was performed twice weekly to monitor ovarian dynamics from 7 ± 2 d after calving until ovulation was confirmed or until 56 DIM, whichever occurred earlier. Cows that did not ovulate by 56 DIM were considered anovular. Measurement of large and small follicles was performed as previously reported by Pierson and Ginther (1984). Ovulation was presumed

Table 1. Ingredients and nutrient composition of dietary treatments[1]

Item	Barley	DDGS[2]
Ingredients, % of DM		
Barley silage	43.0	43.1
Corn grain, rolled	21.6	21.6
Barley grain, rolled	17.3	0.0
Wheat dry distillers grains with solubles	0.0	17.2
Beet pulp	3.2	12.3
Corn gluten meal	8.3	0.0
Vegetable oil	2.4	1.9
Urea	0.3	0.0
Minerals and vitamins	3.9	3.9
Nutrient composition		
DM, %	50.1	50.0
OM, % of DM	89.1	89.1
CP, % of DM	17.3	19.4
NDF, % of DM	27.2	30.5
Starch, % of DM	29.2	19.1
Ether extract, % of DM	3.7	4.4
NFC, % of DM	33.9	38.3

[1]Adapted from Sun and Oba (2014).

[2]DDGS = dried distillers grains with solubles.

if the large follicle (≥10 mm) detected at one examination had disappeared at the subsequent examination and was eventually replaced by a corpus luteum. The simultaneous ovulation of 2 or more follicles in the same cow was considered multiple ovulation.

Fifty-five of 60 cows (1 died, 4 culled) were placed on the Ovsynch protocol (Pursley et al., 1995) and subjected to timed artificial insemination (**TAI**) approximately 73 d postpartum (Figure 1). The protocol included 2 GnRH (100 μg i.m., gonadorelin acetate; Fertiline, Vetoquinol N.A. Inc., Lavaltrie, QC, Canada) treatments 9 d apart and $PGF_{2\alpha}$ (500 μg i.m., cloprostenol, Estrumate, Schering-Plough Animal Health, Pointe-Claire, QC, Canada) given 7 d after the first GnRH. Transrectal ultrasonography was also performed at $PGF_{2\alpha}$ (to confirm CL and dominant follicle), at TAI (to determine preovulatory follicle diameter and to detect early ovulations), and 24 h later to confirm the ovulatory response to the second GnRH treatment (Colazo et al., 2003). Cows that had a preovulatory follicle at TAI and ovulated in response to the second GnRH treatment were considered to have responded to the Ovsynch protocol. Pregnancy was diagnosed 32 d after TAI by ultrasonography and reconfirmed 28 d later.

Blood Sampling

Blood samples were collected from all 60 cows 4 times a week within a 72-h period, with 18-h intervals between samplings from 7 to 84 DIM, to analyze the plasma concentrations of insulin and IGF-1. Blood sampling was performed on wk 1, 2, 3, 4, 6, 8, 10, and 12 after calving (Figure 1). Samples were collected from a coccygeal blood vessel using evacuated Vacutainer tubes containing sodium heparin (Becton Dickinson and Company, Franklin Lakes, NJ) as an anticoagulant. Samples were placed on ice immediately upon collection and centrifuged at 3,000 × *g* for 20 min at 4°C; plasma was harvested and frozen at −20°C until further analysis. At the end of the experimental period, samples were thawed and equal volumes of the plasma samples from each cow were pooled by week and refrozen until further analysis. Plasma concentrations of glucose, fatty acids, and urea were also determined in all cows (Sun and Oba, 2014).

A subset of 24 cows (n = 12 from each dietary group), representing animals in which the first ovulation occurred within 5 wk postpartum and confirmed to have a corpus luteum at 42 DIM, was used to assess the plasma concentrations of glucose, fatty acids, and urea. On the day of follicular fluid collection (see

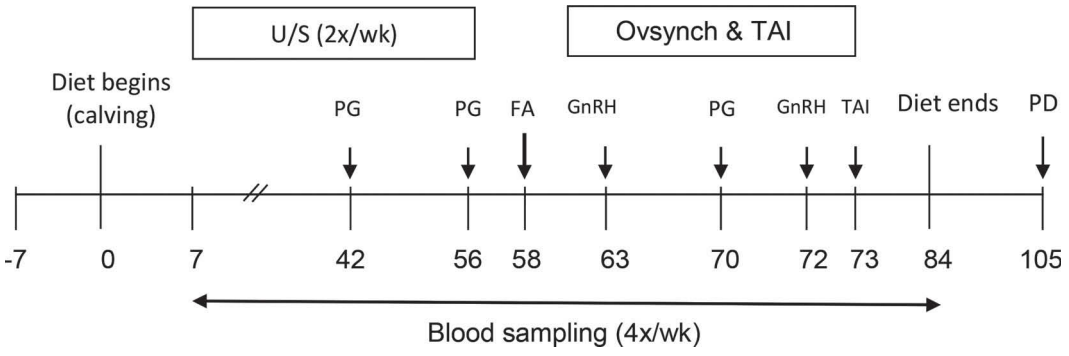

Figure 1. Schematic diagram of treatment schedules for experiment 1. Animals (n = 30/diet) received an experimental diet from the day of calving until 84 DIM. Blood samples were collected on wk 1, 2, 3, 4, 6, 8, 10, and 12 to determine the plasma concentrations of insulin, IGF-1, glucose, fatty acids, and urea. Animals were subjected to 2 $PGF_{2\alpha}$ injections (PG) given 14 d apart at 42 and 56 DIM, respectively, and follicle aspiration (FA) was performed at 58 DIM. Ovsynch protocol was started at 63 DIM and timed AI (TAI) performed at 73 DIM. Ultrasonography (U/S) was performed from 7 to 56 DIM or until the first ovulation. Pregnancy diagnosis (PD) was performed 32 d after TAI in cows that responded to the Ovsynch protocol (n = 40) and were subjected to TAI.

next section), 3 blood samples were collected from the above subset of 24 cows at −30, −15, and 0 min relative to follicle aspiration. The samples were pooled for measuring concentrations of insulin, IGF-I, glucose, fatty acids, and urea, and to determine the relationship between follicular fluid and plasma metabolites. Blood samples were also collected from 40 cows that responded to the Ovsynch protocol, approximately 15 to 18 h after $PGF_{2\alpha}$ and 1 h before TAI to determine plasma estradiol concentrations, as well as 12 d after TAI to determine plasma progesterone concentrations.

Follicular Fluid Collection

The same subset of 24 cows representing those that ovulated within 5 wk postpartum was assigned for follicular fluid collection based on the presence of a corpus luteum at 42 DIM. Ovarian status was synchronized with 2 treatments of $PGF_{2\alpha}$ given 14 d apart at 42 and 56 DIM (Figure 1). Two days after second $PGF_{2\alpha}$ administration (58 DIM), cows were subjected to ultrasound-guided transvaginal follicular aspiration, as previously described by Guzeloglu et al. (2001).

After each collection, the tube containing follicular fluid from the largest follicle was labeled and placed immediately on ice. Samples were centrifuged at 1,500 × *g* for 3 min at 4°C to separate the granulosa cells and cumulus oocyte complexes (**COC**) from the follicular fluid. The follicular fluid supernatant was transferred to cryo-vials (Nalgene, Thermo Fisher, Waltham, MA) for storage at −80°C until further analysis for estradiol, progesterone, insulin, IGF-1, glucose, fatty acids, and urea. The total time elapsed from aspiration to freezing the follicular fluid samples was about 20 min.

The pellet resulting from the centrifugation was resuspended in IVF-TALP (Tyrode's albumin lactate pyruvate- catalog no. IVL02–100mL, Caisson Lab Inc., North Logan, UT) medium, transferred to a petri dish, and examined under a stereomicroscope for COC. After removing any COC that were present, the IVF-TALP medium holding granulosa cells was centrifuged (1,500 × *g*, 3 min, 4°C) and the pelleted granulosa cells were stored in the −80°C freezer until used for gene expression studies.

Reproductive Hormones in Follicular Fluid and Plasma

Estradiol and progesterone concentrations in follicular fluid were determined in all samples to differentiate dominant follicles from atretic and subordinate follicles. A follicle was considered to be estrogenic (dominant) when the estrogen-to-progesterone ratio was greater than 1, whereas for atretic or subordinate follicles the estrogen-to-progesterone was lesser than 1, as reported by Landau et al. (2000) and Leroy et al. (2004). Before analysis, the follicular fluid samples were thawed at 37°C for 10 min and then centrifuged at 3,000 × *g* for 20 min at 4°C, as described by Thangavelu et al. (2008).

Laboratory Analyses

Insulin. A solid phase RIA kit was used to determine the concentrations of insulin (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA) in plasma and follicular fluid (Landau et al., 2000). The kit had been designed for human insulin analyses but was validated for bovine insulin by Reimers et al. (1982). Follicular fluid was diluted 3 times with the zero standards provided with the kit. Plasma samples were assayed in triplicate using 200 μL of plasma. The average intra- and interassay coefficient of variation for plasma were 5.8 and 17.9%, respectively and the assay sensitivity was 1.3 IU/mL. Follicular fluid samples were also assayed in triplicate in one assay with a detection limit of 1.3 IU/mL. The intra-assay coefficient of variation was 8.4%.

IGF-1. Plasma and follicular fluid samples were analyzed using a solid phase, enzyme-labeled chemiluminescent immunometric assay (Immulite, Diagnostic Products Corporation), as previously described by Elmlinger et al. (2005). Both plasma and follicular fluid samples were diluted 1:10 with the pretreatment solution provided with the kit. Intra-assay coefficient of variation was 4.8% for reference sera, whereas it was 4.7% for follicular fluid. The assay sensitivity for IGF-1 was <25 ng/mL.

Glucose, Fatty Acids, and Urea. Plasma and follicular fluid glucose was analyzed using glucose oxidase/peroxidase enzyme (P 7119, Sigma-Aldrich, St. Louis, MO) and o-dianisine-dihydrochloride (F5803, Sigma-Aldrich). The volume of plasma and follicular fluid used for analysis was 10 μL. Fatty acids were determined in 5 μL of plasma and follicular fluid using a commercial kit [NEFA-HR (2), Procedure, Wako Chemicals, Richmond, VA]. The concentrations of urea nitrogen in plasma and follicular fluid were measured enzymatically using 2 and 20 μL volumes, respectively (Fawcett and Scott, 1960).

Estradiol and Progesterone. A solid phase RIA kit (Coat-A-Count, Diagnostic Products Corporation) was used to determine the concentrations of estradiol in plasma and follicular fluid samples by following the manufacturer instructions with the following modification for plasma. The assay was carried out in triplicate using 200 instead of 100 μL of plasma. Samples were analyzed in one plate with an intra-assay coefficient of

Table 2. Details of primers and probes used for reverse transcription PCR analysis

Gene name	Gene accession number	Primers and probes	Primer and probe sequences	Position
Estrogen receptor-β	NM_174051.3	Forward	CCATTGCCAGCCGTCAGT	267–284
		Reverse	GGTTTCACGCCAAGGACTCTT	324–304
		Probe	CTGTATGCAGAACCTC	286–301
LH receptor	U20504.1	Forward	TCCCTGGAGCTGAAGGAAAA	615–634
		Reverse	CGGAAGGCGTCGTTGTG	670–654
		Probe	CACGCCTGGAGAAGA	637–651
Aromatase	U18447.1	Forward	TCGTCCTGGTCACCCTTCTG	1,367–1,386
		Reverse	CGCACCGACCTTGCAAA	1,423–1,407
		Probe	ACGCTTCCACGTGCAG	1,389–1,404
GPX3	NM_174077.3	Forward	AACCCATGAAGGTCCATGACA	533–553
		Reverse	GGCCCCACCAGGAACTTCT	587–569
		Probe	CCGGTGGAACTTT	555–567
GAPDH	U85042.1	Forward	TGCCGCCTGGAGAAACC	715–731
		Reverse	CGCCTGCTTCACCACCTT	771–754
		Probe	CCAAGTATGATGAGATCAA	734–752

variation of 18.6%; the assay sensitivity was 0.75 pg/mL. Follicular fluid samples were diluted 1:500 in PBS (0.01 *M* PBS, pH = 7.5) with 0.1% gelatin as reported by Guzeloglu et al. (2001). A total volume of 100 μL of aliquot per sample was used for the assay. The detection limit for follicular fluid was 6 pg/mL.

A solid phase RIA kit (Coat-A-Count, Diagnostic Products Corporation) was used to determine the concentrations of progesterone in plasma (Colazo and Ambrose, 2011) and follicular fluid samples (Thangavelu et al., 2008). The assays were carried out in triplicate using 100 μL of plasma and follicular fluid. Both the samples were analyzed in one assay with intra-assay coefficient of variation of 3.8 and 5.8% for plasma and follicular fluid, respectively. The assay sensitivity for plasma and follicular fluid was 0.08 and 0.04 ng/mL, respectively.

RNA Isolation, Quantitation, Reverse Transcription, and Real-Time PCR. Only 20 of the 24 cows were used for this part of the study to determine the m-RNA expression levels of *LHr*, estrogen receptor-β, *CYP19*, and *GPx3* genes in granulosa cells. The housekeeping gene was *GAPDH*, as it was reported to be the most stable housekeeping gene for granulosa cells (Machado et al., 2009).

Total RNA was isolated from granulosa cells of preovulatory follicles using TRIzol reagent (Invitrogen, Waltham, MA) following manufacturer's instructions. The homogenized samples were incubated at 25 to 30°C for 20 min instead of 5 min. The RNA quantitation, reverse transcription, and real-time PCR were performed as previously described by Paradis (2009). Details of primers and probes used for reverse transcription PCR analysis are presented in Table 2. Using the cycle threshold (**ΔCt**) method, real-time PCR data for the target gene of interest (**GOI**) was normalized against the respective means of housekeeping gene (i.e., *GAPDH*). The ΔCt value was obtained by subtracting Ct value of *GAPDH* from the respective cow's Ct value of GOI. To determine the gene expression between treatments, the sample that had the most stable expression for all GOI was taken as a calibrator.

Statistical Analyses

Data were analyzed in SAS (version 9.1; SAS Institute Inc., Cary, NC). Significant differences were reported if $P \leq 0.05$; $P > 0.05$ and ≤ 0.10 were considered trends.

All repeated measurements data (plasma insulin, IGF-1, glucose, urea, fatty acids, and estrogen) were analyzed using PROC MIXED. All of the above dependent variables were modeled against diet (i.e., treatment), parity, time (week or day), diet by parity, diet by time, and diet by parity by time. Cow was used as the subject term, whereas repeated measures included time (week or day) of sample collection, and BCS was used as the covariate in the above final model.

Interval from calving to first ovulation was analyzed using the LIFETEST procedure. Events of multiple first ovulation and multiple ovulation at TAI, as well as conception rate at first service were analyzed using the GENMOD procedure. All the previously mentioned binomial variables were modeled against diet, parity, and diet by parity interaction, and model specifications included binomial distribution and logit link function. Other reproductive parameters, such as mean diameter of the largest follicle at first ultrasound, days to reach a dominant follicle of ≥10 mm in diameter, and diameter of preovulatory follicle at TAI, were analyzed using PROC MIXED with diet, parity, and diet by parity interactions and BCS as covariate included in the final model. The model included diet as fixed effect and cow as a random effect. Correlations between diameters of

dominant follicle at first ultrasound, days needed for a dominant follicle to reach ≥10 mm in diameter, and interval from calving to first ovulation were determined using Pearson correlation.

All single measurement data [follicular fluid and pooled plasma samples to measure metabolites (i.e., insulin, IGF-1, glucose, fatty acids, and urea), follicular fluid reproductive hormones (i.e., estradiol and progesterone)] were analyzed using PROC MIXED. Dependent variables were modeled against diet, parity, and diet by parity interactions with BCS included as covariate. The model included diet as a fixed effect and cow as a random effect. Correlations were calculated between plasma and follicular fluid metabolites (i.e., insulin, IGF-1, glucose, fatty acids, and urea) using Pearson correlation. Correlations were also determined between estradiol, insulin, and IGF-1 concentrations in follicular fluid, between follicular fluid estradiol and preovulatory follicle diameter measured before aspiration, and between follicular fluid estradiol and mRNA expression of LH receptor, estrogen receptor-β, and aromatase receptor in GC. The individual fold change for each gene of interest was analyzed using PROC MIXED.

RESULTS

Parity Distribution, DMI, BCS, and Milk Production

Of the 60 cows assigned to the study, 22 were primiparous (n = 11/diet) and 38 multiparous (n = 19/diet). Mean (±SD) prepartum BCS and mean (±SEM) DMI and daily milk yield for the barley and DDGS group cows were 3.7 ± 0.6, 18.0 ± 3.5 kg/d, and 35.1 ± 1.0 kg, respectively ($P > 0.10$). More detailed results relating to DMI, sorting behavior, rumen fermentation, apparent total-tract nutrient digestibility and milk production were reported in a companion paper (Sun and Oba, 2014).

Ovarian Dynamics

The effects of dietary treatment on ovarian function are summarized in Table 3. Regardless of treatment, the mean diameters of the largest ovarian follicle at the first ultrasound examination (7 ± 2 d postpartum) and at TAI (~70 d postpartum) were 11.2 and 16.1 mm, respectively. The mean interval from calving to reach a follicle of ≥10 mm diameter was 11.8 d; none of the 3 parameters mentioned was affected by diet. The interval from calving to first ovulation was not influenced by diet. However, a greater ($P = 0.04$) proportion of cows in the barley group ovulated multiple (≥2) follicles at first ovulation compared with cows in DDGS group, and this difference was evident even later in the postpartum period, as a greater proportion of cows in the barley group tended ($P = 0.10$) to ovulate multiple follicles following first TAI (Table 3).

The interval from calving to first ovulation and the days required for a dominant follicle to attain ≥10 mm diameter were positively correlated (r = 0.26, $P = 0.04$). However, no correlation existed between diameter of dominant follicle at first ultrasound and days to reach ≥10 mm in diameter (r = −0.07; $P > 0.10$), or diameter of dominant follicle at first ultrasound and interval from calving to first ovulation (r = 0.31; $P > 0.10$).

Insulin, IGF-1, and Metabolites in Plasma

Overall mean insulin concentration was higher in cows fed barley compared with those fed DDGS (2.5 ± 0.3 vs. 1.6 ± 0.3 IU/mL; $P < 0.01$). Weekly mean insulin concentrations in plasma increased with time from wk 1 to 12 postpartum ($P < 0.01$), and tended ($P = 0.08$) to be higher in the cows in barley group than in those in the DDGS group from wk 6 to 12 postpartum (Figure 2). However, neither parity nor parity by diet interaction affected insulin concentrations in plasma.

Table 3. Effect of dietary starch on ovarian follicular dynamics

Reproductive parameter	Barley (n = 30)	Dry distillers grains with solubles[1] (n = 30)	*P*-value
Mean diameter of largest follicle at first ultrasound scan[2] (mm)	11.3 ± 0.4	11.2 ± 0.4	0.85
Days to reach a follicle of ≥10 mm in diameter	12.4 ± 1.1	11.1 ± 1.1	0.24
Interval from calving to first ovulation (d)	33.9 ± 2.3	30.7 ± 2.6	0.75
Multiple first ovulations [no. (%)]	12/30 (40)	6/30 (20)	0.04
Multiple ovulations at first timed AI[2] [no. (%)]	4/19 (21)	2/21 (10)	0.10
Diameter of preovulatory follicle at first timed AI[2] (mm)	16.3 ± 0.7	16.0 ± 0.7	0.59
Conception rate at first timed AI[2] [no. (%)]	7/19 (37)	7/21 (33)	0.97

[1]Lower starch diet.

[2]Only in cows that responded to Ovsynch protocol and were subjected to timed AI (n = 40).

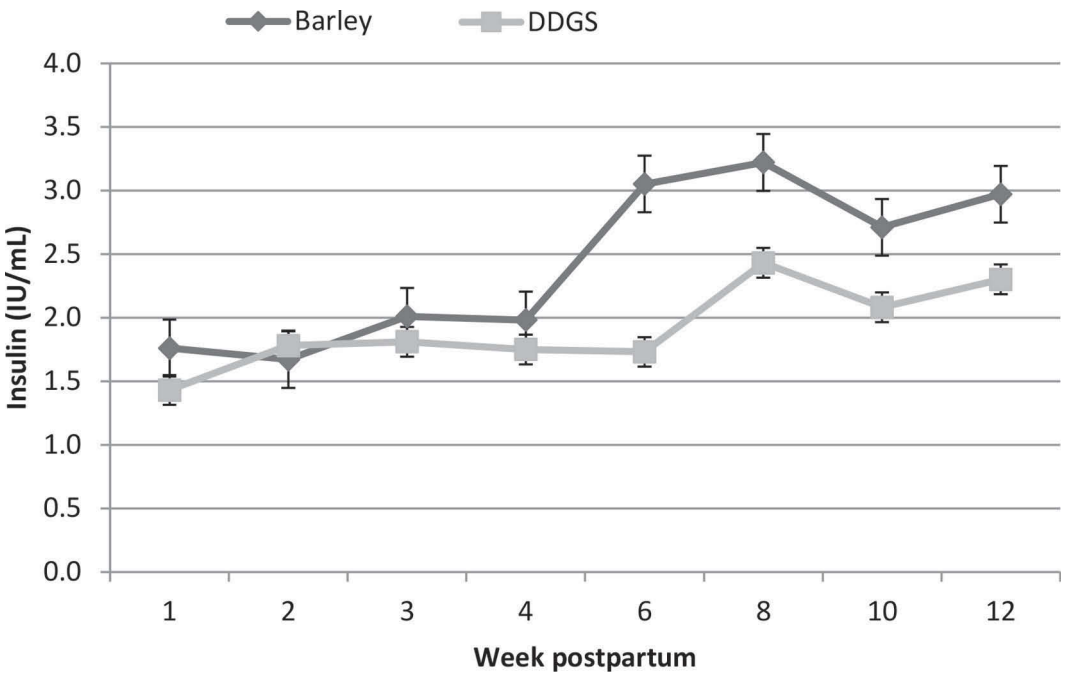

Figure 2. Plasma insulin concentrations (±pooled SEM) from wk 1 to 12 postpartum. Cows (n = 30/diet) were fed rations containing either barley or dry distillers grains with solubles (DDGS) from calving to 84 DIM. Cows fed barley tended to have higher plasma insulin concentration compared with those in DDGS group from wk 1 to 12 postpartum ($P = 0.08$).

No dietary effects were evident for plasma IGF-1 concentrations (70.7 ± 4.1 vs. 75.9 ± 4.1 ng/mL; $P > 0.10$) between cows fed DDGS and those fed barley. Although mean IGF-1 concentrations increased from wk 1 to 6 postpartum ($P = 0.01$; Figure 3), no diet by time interaction ($P > 0.10$) was noted on IGF-1 concentrations during that period. Parity significantly influenced plasma IGF-1; multiparous cows had lower concentration of IGF-1 than primiparous cows (62.2 vs 84.4 ng/mL; $P < 0.01$). Mean plasma insulin (2.4 ± 0.27 IU/mL) and IGF-1 concentrations (76.0 ± 5.5 ng/mL) did not differ ($P > 0.10$) between cows with single and multiple ovulations.

Plasma concentrations of glucose, fatty acids, and urea from all cows used in this study have been reported elsewhere (Sun and Oba, 2014). Diets did not alter glucose and urea ($P > 0.10$) concentrations in plasma; however, cows fed DDGS tended ($P = 0.08$) to have higher concentrations of fatty acids than those fed barley.

Hormones and Metabolites in Plasma and Follicular Fluid

Diet, parity, or diet by parity interaction did not influence plasma concentrations of insulin, IGF-1, glucose, fatty acids, or urea in the subset of cows (Table 4). Similarly, diet, parity, and diet by parity interaction did not affect follicular fluid concentrations of insulin, glucose, and urea. Whereas diet had a significant influence on fatty acid concentrations in follicular fluid, with cows fed barley having lower fatty acids than those fed DDGS (Table 4); parity or diet by parity interactions did not affect fatty acid concentrations in follicular fluid. Follicular fluid concentrations of IGF-1 were higher in cows fed barley grain compared with those fed DDGS (Table 4) and primiparous cows had higher IGF-1 in follicular fluid than in multiparous cows (105.1 ± 12.4 vs 72.1 ± 9.9 ng/mL). Cows fed DDGS had higher mean plasma fatty acid concentrations compared with those fed barley grain (182 ± 22.9 vs. 132 ± 22.9; $P = 0.04$), which progressively declined in both dietary groups from wk 1 to 12 postpartum (Figure 4).

In this subset of 24 cows, mean estradiol and progesterone concentrations in follicular fluid were 1174.0 ± 159.0 and 69.8 ± 7.7 ng/mL, respectively. The estradiol-to-progesterone ratio was greater than 1.0 in all follicular fluid samples.

Estradiol and Progesterone in Plasma

Estradiol concentrations were not affected ($P > 0.10$) by diet, parity, or diet by parity interactions either at 15 to 18 h after $PGF_{2\alpha}$ administration or at 1 h before TAI. However, the interaction of diet by parity had a

Table 4. Concentrations of metabolites in plasma and follicular fluid, and their correlation in a subset of 24 cows on the day of follicle aspiration[1]

	Plasma			Follicular fluid			
Metabolite	Dry distillers grains with solubles	Barley	*P*-value	Dry distillers grains with solubles	Barley	*P*-value	r
Insulin (IU/mL)	3.1 ± 0.4	3.1 ± 0.5	0.89	10.7 ± 0.2	10.6 ± 0.3	0.65	0.12
IGF-1 (ng/mL)	107.0 ± 11.5	115.0 ± 12.7	0.65	69 ± 1.0	108 ± 12.4	0.02	0.08
Glucose (mg/dL)	71.2 ± 2.4	70.1 ± 2.6	0.77	56.5 ± 1.2	56.7 ± 1.5	0.93	−0.20
Fatty acids (mEq/L)	537.0 ± 99.0	373.0 ± 108.5	0.27	222 ± 19.0	149.0 ± 23.7	0.02	0.36
Urea (mg/dL)	5.8 ± 0.6	7.2 ± 0.7	0.14	70.0 ± 1.2	70.1 ± 2.4	0.97	0.20

[1]Cows were fed rations containing either dry distillers grains with solubles or barley from the day of calving; the follicular fluid and plasma samples were collected at 58 DIM. Plasma samples were obtained 30, 15, and 0 min before follicular fluid collection and the mean of 3 samples were used to evaluate the correlation between follicular fluid and plasma metabolites concentration.

significant ($P = 0.04$) influence on plasma progesterone concentration 12 d after TAI, with multiparous cows of the DDGS group having higher progesterone than primiparous cows (7.9 vs 5.4 ng/mL).

Relationship Between Hormones and Metabolites in Plasma and Follicular Fluid

No relationships were found between follicular fluid and plasma concentrations of insulin, IGF-1, glucose, fatty acids, and urea (Table 4). Mean estradiol and insulin concentrations in follicular fluid were positively correlated (r = 0.47, $P = 0.02$), but estradiol and IGF-1 concentrations were not correlated (r = 0.19, $P > 0.10$).

Follicle Size and Follicular Fluid Estradiol Concentrations

A positive correlation existed between the preovulatory follicle diameter measured before follicle aspiration and follicular fluid estradiol concentrations (r = 0.44, $P = 0.04$). However, DDGS and barley diets did not influence the size (diameter) of the preovulatory follicles (15.5 ± 0.68 vs. 14.3 ± 0.68 mm; $P > 0.10$).

Gene Expression in Granulosa Cells

Diets did not influence the expression of any of the genes of interest in the granulosa cells of preovulatory

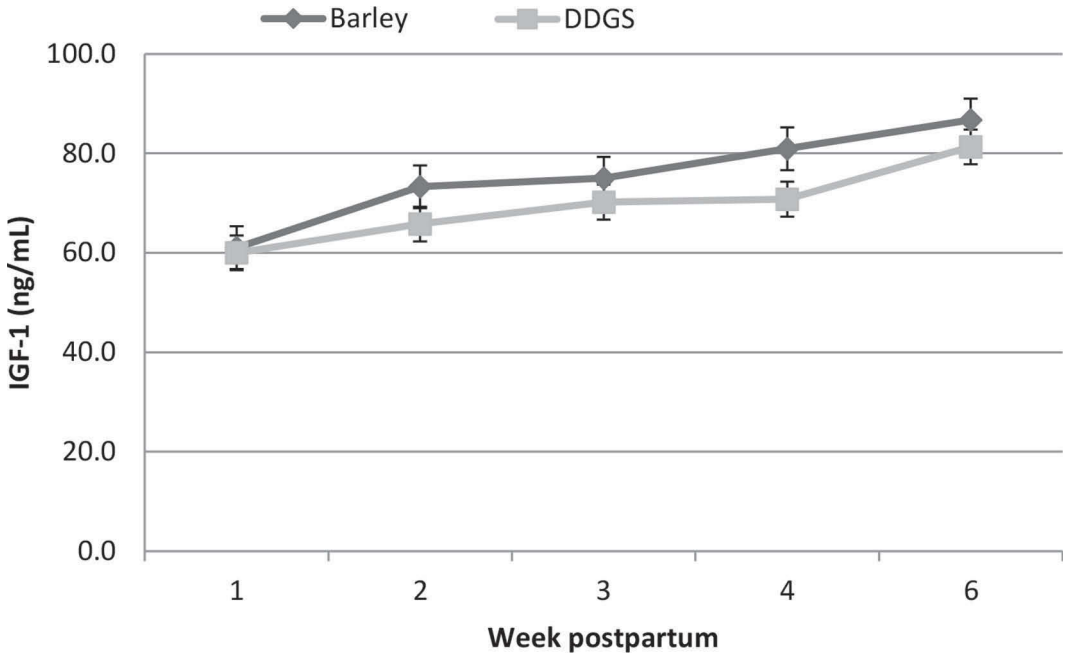

Figure 3. Effect of diet on plasma IGF-1 concentrations in all 60 cows from wk 1 to 6 postpartum (n = 30/diet). Diet had no effect on plasma IGF-1 concentrations ($P = 0.36$; pooled SEM = 5.75). However, a time effect on IGF-1 concentrations was evident ($P < 0.01$). Cows were fed 2 levels of starch [barley and dry distillers grains with solubles (DDGS) at 29 and 19%, respectively] from the day of calving to 84 DIM. Error bars represent pooled SEM.

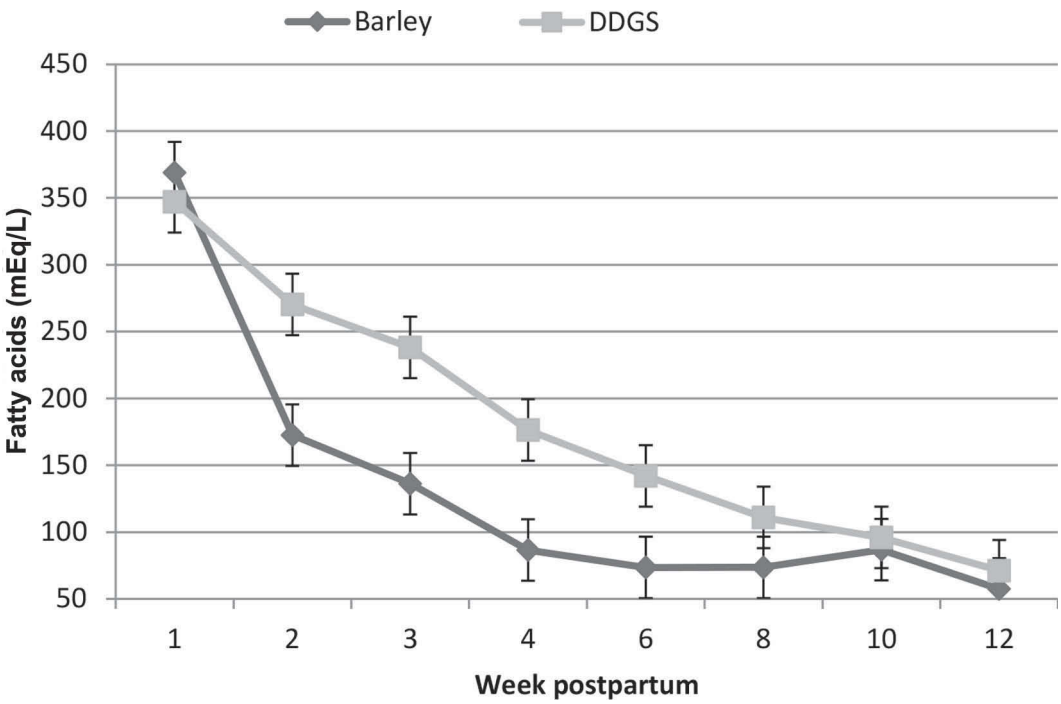

Figure 4. Plasma fatty acid concentrations (±pooled SEM) from wk 1 to 12 postpartum in a subset of 24 cows representing those that ovulated within the first 5 wk postpartum. Cows (n = 12/diet) were fed rations containing either dry distillers grains with solubles (DDGS) or barley from calving to 84 DIM. Cows in barley group had a lower mean fatty acid concentration ($P = 0.04$) compared with those in the DDGS group. Plasma fatty acid concentrations decreased with time ($P < 0.01$) from wk 1 to 12 postpartum. No diet × parity interaction was found ($P > 0.10$).

follicles (Table 5). No associations were found between follicular fluid estrogen concentration and gene expression of LH receptor, estrogen receptor-β, or aromatase receptor in granulosa cells of preovulatory follicle. However, a strong positive correlation existed between mRNA expression of aromatase receptor and estrogen receptor-β in granulosa cells (r = 0.98, $P < 0.0001$).

DISCUSSION

This study is unique in that it investigated the effects of reducing dietary starch content by replacing barley grain with wheat DDGS on ovarian function and hormones or metabolites in plasma and follicular fluid in dairy cows during early lactation. Despite the differences in dietary starch content between the 2 experimental diets, the interval from calving to first ovulation was not affected, contradicting some previous studies. In this regard, Gong et al. (2002) reported that a significantly greater proportion (90 vs. 50%) of dairy cows ovulated before 50 d postpartum when diets contained 26 versus 10% starch. Similarly, in previous work from our group (Dyck et al., 2011), lactating dairy cows fed diets containing 27% starch had a shorter interval (31 d) from calving to first ovulation than cows fed diets containing either 25 (43 d) or 23% (38 d) starch. The absence of any difference in the interval from calving to first ovulation between the 2 dietary groups in the present study may be partially explained by the lack of differences observed in mean plasma insulin concentrations from wk 1 to 4. Plasma insulin concentration differed between diets only at wk 6 (42

Table 5. Summary of results showing the fold change in the expression of target genes in the granulosa cells of preovulatory follicle

Gene name (abbreviation)	Barley (n = 12)	DDGS[1] (n = 12)	SEM	*P*-value
LH receptor (*LHr*)	0.1	0.2	0.2	0.52
Estrogen receptor-β (*ER-β*)	0.2	0.8	0.5	0.23
Aromatase receptor (*CYP 19*)	0.2	0.3	0.2	0.72
Glutathione peroxidase 3 (*GPX 3*)	0.4	0.4	0.2	0.94

[1]DDGS = dried distillers grains with solubles.

d) postpartum, approximately 10 d after the mean interval from calving to first ovulation, which was 32.3 d. In contrast, Gong et al. (2002) observed a greater difference in mean plasma insulin concentration (0.44 vs. 0.22 ng/mL) from the beginning of their study until 50 d postpartum between cows fed diets with 26 versus 10% starch.

The mean interval from calving to first ovulation tended to be longer for primiparous cows (36 d) than that of multiparous cows (30 d), which agrees with that of Dyck et al. (2011), who reported that first ovulation after calving occurred 13 d earlier in multiparous cows than in primiparous cows (32 vs. 45 d). The delay in first ovulation in primiparous cows could be due to higher energy demands for both growth and lactation than in multiparous cows (Tanaka et al., 2008).

Whereas twinning could be profitable in beef cattle production systems (de Rose and Wilton, 1991; Small et al., 2008), in dairy cattle twinning is considered undesirable, as it often increases the risk of perinatal loss and postpartum disease and increases average days open and services per conception during the subsequent lactation (Andreu-Vázquez et al., 2012). Although twinning rates in dairy cows range from 2.4 to 5.6% (Kinsel et al., 1998; Fricke and Wiltbank, 1999; Colazo and Ambrose, 2014), a high incidence of multiple ($\geq$2) ovulations in lactating dairy cows at first ovulation after calving is commonly reported (Wiltbank et al., 2000; López-Gatius et al., 2005), with an incidence of up to 61% reported in one study (Gümen et al., 2005). Whereas the incidence of multiple ovulations is considerably lower at the end of the voluntary waiting period, it reportedly ranges from 11.4 (Colazo and Ambrose, 2014) to 15.5% (López-Gatius et al., 2005). Even though our study was not designed to test differences in multiple ovulations, we found that the incidence of multiple ovulations is higher in cows fed higher levels of dietary starch. In this regard, numerous factors have been identified as possible contributors to multiple ovulations and twinning in dairy cattle, including genetics, age of dam, parity, use of hormones, length of dry period, and milk production (Kinsel et al., 1998; Fricke and Wiltbank, 1999; Wiltbank et al., 2000; Gümen et al., 2005; López-Gatius et al., 2005), with the latter identified as the single largest contributor to increased rates of double ovulations and twinning in lactating dairy cows (Kinsel et al., 1998; Wiltbank et al., 2000). Yet, a nutritional link to multiple ovulations in dairy cows is rarely reported. In a previous report from our group (Dyck et al., 2011), albeit with a smaller sample size than in the present study, cows fed higher-starch diets (27 vs. 23% starch) were at a greater risk for multiple ovulations (46 vs. 0% at first ovulation), suggesting high dietary starch content as a new risk factor for multiple ovulations in the postpartum dairy cow.

We emphasize that neither the study by Dyck et al. (2011) nor the present study was adequately powered to test a binomial variable such as the incidence of multiple ovulations. More importantly, the finding that multiple ovulations were reduced in cows fed low-starch diets was incidental and not a preplanned objective in both studies. It is noteworthy that when only the main effect of diet was modeled against the dependent variable (i.e., multiple ovulation), no difference in the incidence of multiple ovulation was found. However, when parity and parity by diet interactions were added to the model, the incidence of multiple ovulation significantly differed between the 2 rations. Significant parity and diet by parity interactions were reported on DMI, BW, and BCS in our companion paper (Sun and Oba, 2014). In the present study, parity had a significant effect on plasma IGF-1 concentrations when all cows were considered; furthermore, parity is known to have significant effects on the incidence of multiple ovulations (Wiltbank et al., 2000). For those reasons, diet, parity, and parity by diet interactions were included in the final statistical model in all analyses. The present finding of reduced multiple ovulations at first postpartum ovulation in cows fed a wheat DDGS-based ration than in cows fed barley grain calls for further investigations on the link between dietary starch and ovulation rate in the postpartum dairy cow.

The increased occurrence of multiple ovulations in barley group cows in the present study could have been associated with a relative increase in plasma insulin and follicular fluid IGF-1 concentrations compared with those in wheat DDGS group. In this regard, Echternkamp et al. (1990) attributed the increased twinning rates observed in beef cattle to higher circulating IGF-1 concentrations, and Butler et al. (2003) indicated that increased plasma concentrations of insulin would increase the expression of growth hormone receptors in liver, which in turn would increase the production of IGF-1. Whereas we attributed the lower incidence of multiple ovulations in DDGS fed cows to its reduced starch content, it is not known if other factors, such as a slightly higher CP and NDF in the DDGS ration, had a role in altering ovarian function.

Our study was not adequately powered to assess the incidence of multiple ovulation; hence, the findings should be interpreted with caution. Nevertheless, our results support that of Dyck et al. (2011), strengthening the potential link between high intake of dietary starch and the incidence of multiple ovulation. Larger experiments with adequate statistical power are needed to confirm the present findings.

Follicular growth and the number of ovulations were positively influenced by concentrations of plasma insulin and IGF-1 (Armstrong et al., 2002). Those same authors found that insulin concentrations increased with dietary energy and reportedly mediated the dietary effects on ovarian function. In the present study, cows on the barley diet (29.2% starch) had higher mean insulin concentrations than those on the DDGS diet (19.1% starch) starting from wk 6 to 12 postpartum, but not earlier; whereas Gong et al. (2002) found that cows fed a 26% starch diet had higher plasma insulin concentrations than in those fed 10% starch diet from the beginning of experimental period until 50 d postpartum. More recently, McCarthy et al. (2015) reported elevated insulin concentrations in cows fed high- (26.2%) versus low-starch (21.5%) diets offered during the first 3 wk postpartum in dairy cows. Higher insulin concentrations were also reported when starch was infused into the rumen or abomasum of dairy cows (Knowlton et al., 1998). In contrast, Dyck et al. (2011) found no difference in circulating insulin between dairy cows fed diets containing 23 versus 27% starch. Similar to the findings of Dyck et al. (2011), no treatment effects were evident for plasma IGF-1 and glucose concentrations in the present study.

Concentrations of fatty acids decreased from wk 1 to 12 more rapidly in cows that were in the barley group (Figure 4) in our study. Although this finding is based on a subset of only 24 cows, results from all cows also followed a somewhat similar trend, with overall lower mean fatty acid concentrations in cows fed barley than in those fed DDGS (142 vs. 173 mEq/L, $P = 0.08$; Sun and Oba, 2014). This finding also agrees with that of McCarthy et al. (2015), who reported lower fatty acid concentrations in cows fed a high-starch diet than in those fed a low-starch diet during the first 3 wk postpartum. Knowlton et al. (1998) also reported lower fatty acid concentrations when starch was infused either into the rumen or abomasum. No differences in plasma glucose concentrations were found from wk 1 to 12 postpartum, which is in contrast to the findings of Dyck et al. (2011), where plasma glucose decreased from calving to first week postpartum and then increased until 70 d postpartum without any treatment effect. McCarthy et al. (2015) also reported an increase in plasma glucose concentrations during the first 3 wk postpartum in cows fed a high- versus a low-starch diet.

Diets did not affect follicular fluid insulin concentrations. This is in contrast to the findings of Landau et al. (2000), who reported that cows fed corn grain had 26% higher insulin concentrations in follicular fluid than those fed corn gluten meal ($P < 0.05$). Cows fed the barley diet had higher concentrations of IGF-1 in follicular fluid than those in DDGS group in the present study. We are not aware of another study that determined IGF-1concentrations in follicular fluid of lactating cows fed diets with different starch content. Kendrick et al. (1999) found that cows fed high-energy diets had higher mean follicular fluid IGF-1 concentrations from d 30 to 100 postpartum than those fed low-energy diets. Schoppee et al. (1996) reported that chronic feed restriction in cattle decreased the intrafollicular IGF-1 concentrations, indicating that dietary restriction negatively affects IGF-1 concentrations in follicular fluid. The lack of difference in plasma IGF-1 between the dietary groups, but a significantly higher concentration of IGF-1 in follicular fluid of barley-fed cows in the present study, suggests a preferential intrafollicular accumulation of IGF-1, possibly to support cellular function and proliferation within the dominant follicle. Considering the strong association between increased IGF-1 and twinning in cattle (Echternkamp et al., 2004), we attributed the increased rate of multiple ovulations in cows fed a high-starch diet in the present study to the high intrafollicular IGF-1 concentrations.

Leroy et al. (2004) reported that the concentrations of glucose, fatty acids, and urea in serum were reflected in follicular fluid and also found that oocyte and granulosa cells from dominant follicles were protected from exposure to lower glucose and higher fatty acid concentrations, as these concentrations may contribute to detrimental effects on oocyte and reduces fertility in high-yielding dairy cows. Echternkamp et al. (1990) reported that the IGF-1 concentration in follicular fluid from larger bovine follicles and that in blood were positively correlated. Because of these previous reports, we were interested in knowing the associations among metabolites including insulin in plasma and follicular fluid. However, no significant correlations were evident for any of the plasma and follicular fluid metabolites.

Spicer and Echternkamp (1995) reported that both insulin and IGF-1 stimulate estradiol production by granulosa cells, but insulin may be a more potent stimulator in cattle. Interestingly, we found a positive relationship between follicular fluid concentrations of insulin and estradiol, which is in agreement with the findings of Landau et al. (2000), who reported a similar significant association regardless of dietary treatment.

Diets did not influence expression of the genes for LH receptor, estrogen receptor-β, aromatase receptor, and glutathione peroxidase receptor 3 in granulosa cells of preovulatory follicles. In the current study, no correlation was observed between LH mRNA expression in granulosa cells and preovulatory follicle diameter (mean diameter was 15.9 ± 1.4 mm). Beg et al. (2001) reported that the expression of LH mRNA was positively associated with the diameter of the follicle as the mRNA expression was higher in the dominant follicle

with the mean diameter of 10.8 mm; however, their study was independent of dietary effects. They also reported that an increase in LH mRNA expression would increase the LH receptor in granulosa cells, as the latter plays an important role in the events associated with follicular deviation or selection.

During follicular deviation, estradiol production in dominant follicle depends on aromatase enzyme activity in the granulosa cells (Ginther et al., 1996). No correlation was observed between aromatase gene expression and follicular fluid estradiol concentration in our study, which agrees with the findings of Tian et al. (1995), where aromatase mRNA was not expressed in dominant follicles even though an increase in serum estradiol and LH concentrations was noted. Therefore, the expression of aromatase mRNA was not a limiting factor in follicular fluid estradiol synthesis (Calder et al., 2001). However, we found a positive association between aromatase mRNA expression levels and estrogen receptor-β (**ER-β**). Aromatase enzyme is necessary for the production of estradiol in follicular fluid during follicular deviation (Ginther et al., 1996), and the role of estradiol may be primarily mediated by ER-β. In the present study, we measured the expression of ER-β in granulosa cells because it is the predominant estrogen receptor found in the ovary compared with estrogen receptor-α (Byers et al., 1997).

We found no correlation between follicular fluid estradiol concentrations and mRNA expression levels of LH receptor, ER-β, and aromatase receptor. Conversely, Evans et al. (2004) found a positive association between estradiol concentrations in follicular fluid and m-RNA expression for the 3 GOI mentioned in dominant but not in subordinate follicles, implying that estradiol plays an important role in continued growth of dominant follicles after follicular selection or deviation.

CONCLUSIONS

In summary, diets containing different levels of starch did not affect the interval from calving to first ovulation or granulosa cell gene expression. However, reducing dietary starch by partial replacement of grain with wheat DDGS, a high-fiber, high protein, low-starch byproduct of the ethanol industry, increased fatty acids in follicular fluid and reduced concentrations of insulin in plasma, IGF-1 in follicular fluid, and the incidence of multiple ovulations in postpartum dairy cows.

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